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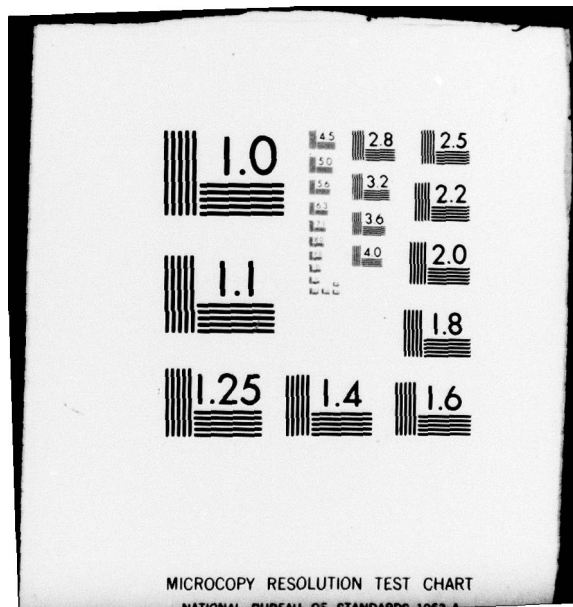
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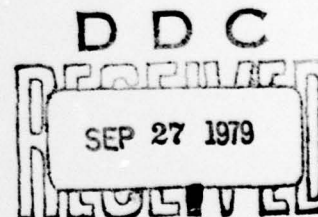
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PHASE REPORT  
AIRTASK NO. WF12-100-000  
Work Unit No. ZT201

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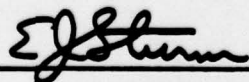
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4. TITLE (and Subtitle) Studies on PGB Sub X. Isolation of a PGB With Reduced Inhibitor Content.	5. TYPE OF REPORT & PERIOD COVERED Phase Report		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR H. W./Shmukler, E./Soffer, S.F./Kwong, M.G./Zawryt W./Feely	8. CONTRACT OR GRANT NUMBER(s)		9. PROGRAM ELEMENT/PROJECT, TASK AREA & WORK UNIT NUMBERS AIRTASK NO. WF12 100 000 Work Unit No. ZT201
10. PERFORMING ORGANIZATION NAME AND ADDRESS Naval Air Development Center Aircraft & Crew Systems Technology Directorate Warminster, PA 18974	11. CONTROLLING OFFICE NAME AND ADDRESS Office of Naval Research Department of the Navy Arlington, VA 22217		12. REPORT DATE
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18. SUPPLEMENTARY NOTES 8 Aug 79			
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) PGB Isolation Reduced Inhibitor PGBX			
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) PGB <sub>x</sub> was separated into two fractions by dialysis against pH 6.5 phosphate buffer. The retentate, which amounted to 90% of the total PGB <sub>x</sub> , showed <u>in vitro</u> PGB <sub>x</sub> effects similar to the unfractionated PGB <sub>x</sub> , i.e., activation at low concentration, inhibition at high concentration. The dialysate fraction showed primarily PGB <sub>x</sub> activation with only minimal inhibition properties. It is concluded that the activation and inhibition effects of the PGB <sub>x</sub> complex are caused by different molecular species in the complex.			

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## INTRODUCTION

The role of  $\text{PGB}_x^*$  in the in vitro recovery of oxidative phosphorylation of degraded aged rat liver mitochondria was first reported by Polis et al (1). In a later publication the details of the in vitro assay were described fully (2, 6). In both these reports the restoration of oxidative phosphorylation was shown to be directly dependent upon the concentration of  $\text{PGB}_x$  in the range of 2-10  $\mu\text{g}$  per 4 mg of isolated rat liver mitochondria. In later experiments, Polis (3) showed that at higher concentrations of  $\text{PGB}_x$  the reversal of oxidative phosphorylation was decreased and eventually reached the level of the control, i.e., no net phosphorylation. Similar results were found by Devlin (4) using a modification of the in vitro test system in that the mitochondria were only partially degraded. In this system, high concentrations of  $\text{PGB}_x$  inhibited the oxidative phosphorylation reactions below that of the control.

This biphasic response of  $\text{PGB}_x$  on mitochondrial oxidative phosphorylation, i.e., activation at low concentrations and inhibition at high concentrations, suggested a metabolic role for  $\text{PGB}_x$  in terms of a feedback mechanism that could regulate the rate of formation of biological energy in mitochondria, similar to hormonal control mechanisms. This would imply that the dual in vitro effects of  $\text{PGB}_x$  must be inherent in a single molecular species. Alternatively, these two effects may be associated with different factors making up the  $\text{PGB}_x$  complex currently being prepared. In view of the known heterogeneity of the  $\text{PGB}_x$  complex, it was deemed important to determine if the activating and inhibiting effects of  $\text{PGB}_x$  were separable. This report describes the separation of  $\text{PGB}_x$  into fractions of different molecular weights that exhibit the same degree of activation but different degrees of inhibition.

## EXPERIMENTAL

$\text{PGB}_x$  acid (preparation #27), prepared by the method of Polis et al (2), was converted to the sodium salt as described below.  $\text{PGB}_x$  acid was first dissolved in ethanol and then diluted with water to 60% ethanol. Dilute aqueous NaOH was added to neutralize this solution to pH 7.2-7.4. The solution was frozen at  $-70^\circ$  and then lyophilized to yield the dried sodium salt of  $\text{PGB}_x$ .

The  $\text{PGB}_x$  in vitro effect was assayed by the method described previously (2); however, the system was modified to include the assay for the inhibition effect as well as the activation effect. This modification consisted of testing the effect of  $\text{PGB}_x$  on oxidative phosphorylation of aged degraded rat liver mitochondria over a concentration range of 2-80  $\mu\text{g}$   $\text{PGB}_x$  per 4 mg of mitochondria. Sufficient data points were used to define the rising side of the activation curve (2,4,6,7,10  $\mu\text{g}$   $\text{PGB}_x$ ) and the falling side (20,30,50,80  $\mu\text{g}$   $\text{PGB}_x$ ). The degree of activation ( $K_a$ ) and inhibition ( $K_i$ ) were quantified as described previously (5).

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\*Small amounts of  $\text{PGB}_x$ , for investigative purposes, are available from the authors upon application.

The dialysis experiments were carried out by dissolving the sodium  $\text{PGB}_x$  in the minimum amount of dialysis buffer ( $< 5$  ml) needed to dissolve the salt. The  $\text{PGB}_x$  solution was then placed inside a dialyzer tube, which was secured at both ends and then immersed into 1000 ml of buffer at  $4^\circ$ . By continually stirring the external buffer solution, dialysis equilibrium was attained more rapidly. After 24 hours, the dialysate was removed and fresh buffer added. Dialysis was allowed to proceed an additional 24 hours. The dialysates were then combined, acidified to pH 3.0 with dilute perchloric acid and the  $\text{PGB}_x$  acid extracted into isobutanol. The isobutanol layer was washed 3 times with water, and the  $\text{PGB}_x$  acid in the isobutanol layer was dried by flash evaporation at  $50^\circ$ . The residue was dissolved in ethanol and stored at  $3^\circ$  until used. The fraction of  $\text{PGB}_x$  that did not pass through the dialysis tubing also was extracted, dried, and stored as described above.

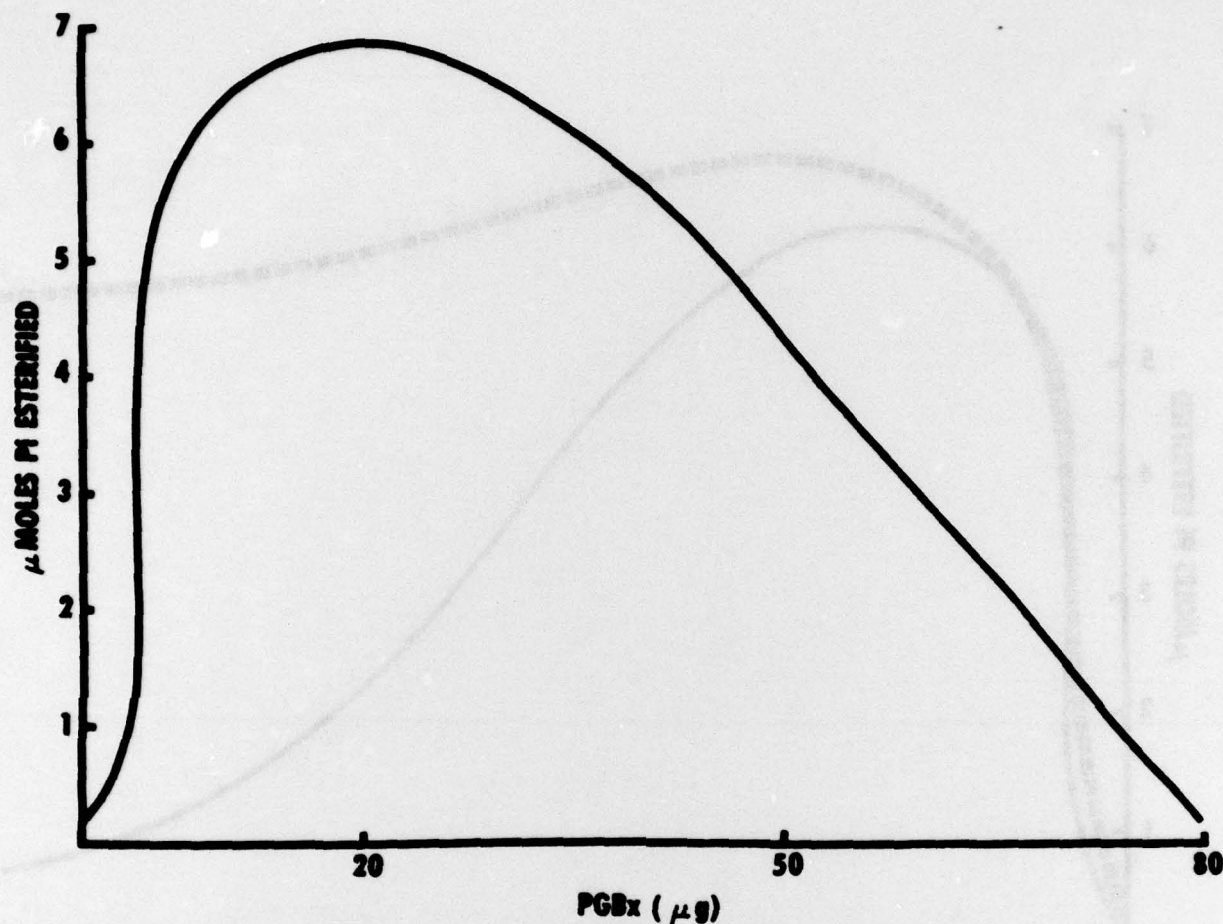
#### RESULTS AND DISCUSSION

In a previous study (5) it was found that the dialysis of  $\text{PGB}_x$  against neutral aqueous phosphate buffers separated the  $\text{PGB}_x$  into two fractions: dialysate and retentate. On the basis of the manufacturer's specifications for the molecular weight separations obtainable with this dialysis tubing, the dialysate was assigned a molecular weight of less than 12000 daltons and the retentate a value of more than 12000 daltons. In contrast, the molecular weight measurements by vapor phase osmometry of the retentate fraction dissolved in methanol was 2500. This discrepancy between the values for the molecular weight of the retentate fraction, as measured by vapor pressure osmometry and by the dialysis experiments, cannot be explained at this time.

In the dialysis study previously reported (5), it was shown that the  $\text{PGB}_x$  activation effect of the dialysate and retentate were almost identical regardless of the pH of the dialysis separation. In this study, the  $\text{PGB}_x$  assay was extended to include the inhibition effect as well. Figure 1 shows the  $\text{PGB}_x$  assay as a function of the concentration of added  $\text{PGB}_x$ . At low  $\text{PGB}_x$  concentrations (2-10  $\mu\text{g}$ ) the amount of phosphorylation increases with increasing amounts of  $\text{PGB}_x$ . At medium  $\text{PGB}_x$  concentrations (13-20  $\mu\text{g}$ ) the phosphorylation is constant with increase in  $\text{PGB}_x$ . In contrast, at high  $\text{PGB}_x$  concentrations (20-80  $\mu\text{g}$ ) the phosphorylation decreased with increasing amounts of  $\text{PGB}_x$ . The fractions from the dialysis experiments at pH 7.8, pH 7.2 and pH 6.5 (5) were assayed for both effects. In general, the dialysates and retentates from the pH 7.8 and pH 7.2 gave  $\text{PGB}_x$  effects quite comparable to the standard  $\text{PGB}_x$ , so that at these pH's the dialysis apparently separated  $\text{PGB}_x$  into fractions according to molecular size with no apparent separation of the activation and inhibition effects. However, after dialysis at pH 6.5 the dialysate had a markedly reduced amount of inhibitor content, while the activator content of both fractions were almost identical.

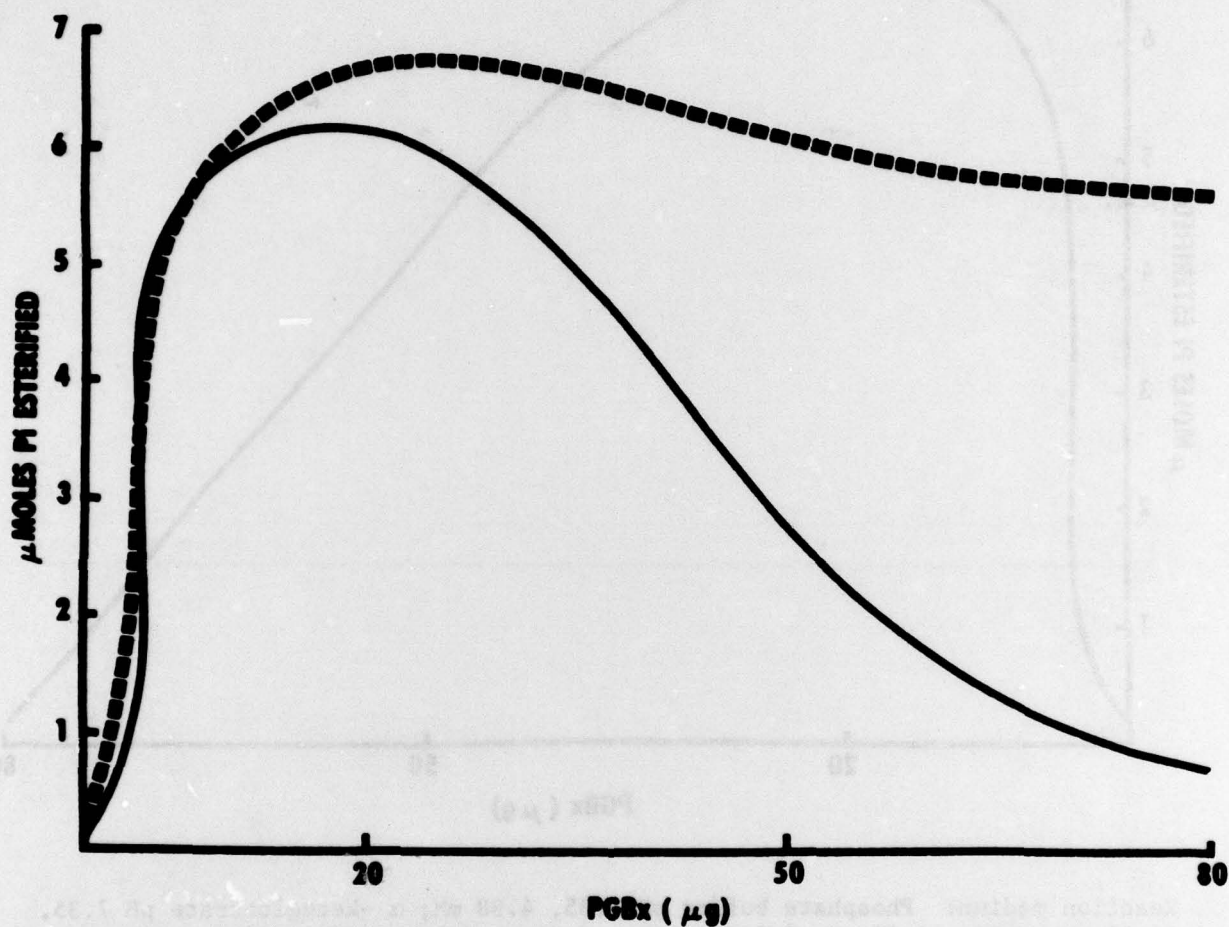
The  $\text{PGB}_x$  activity vs concentration curves of the dialysate and retentate shown in figure 2 demonstrate the separation of  $\text{PGB}_x$  into fractions of different molecular weights, with similar amounts of  $\text{PGB}_x$  activator and different amounts of inhibitor factor. Clearly the pH 6.5 dialysate yields a  $\text{PGB}_x$  fraction with a low molecular weight and a marked reduction in the  $\text{PGB}_x$  inhibitor content.





Reaction medium: Phosphate buffer pH 7.35, 4.98 mM;  $\alpha$ -ketoglutarate pH 7.35, 14.93 mM;  $\text{MgSO}_4$ , 4.98 mM; 3-5 day old rat liver mitochondria, 4 mg protein (containing sucrose and EDTA to yield 5.97 mM and 0.010 mM respectively); and water to 2.01 ml. When  $\text{PGB}_x$  was added, the water was reduced an equivalent amount. The mixture was shaken in covered beakers maintained at 27°. At the end of 8 minutes AMP, ADP, KCl and serum albumin were added to a final concentration of 2.27 mM, 2.27 mM, 45.45 mM and 0.68 mg/ml respectively. The reaction was allowed to proceed for 20 minutes and stopped by the addition of 0.5 ml of 31%  $\text{HClO}_4$ . Esterified phosphate was determined as described previously (2).

Figure 1 - The Effect of Standard  $\text{PGB}_x$  on the in vitro Oxidative Phosphorylation of Degraded Aged Rat Liver Mitochondria



Solid curve, retentate; dashed curve, dialysate. Assay conditions are the same as figure 1.

Figure 2 - The Effect of Additions of  $PGB_x$  Fractions Separated by Dialysis at pH 6.5 on the in vitro Oxidative Phosphorylation of Degraded Aged Rat Liver Mitochondria

These results are summarized in table I, which shows the distribution of the weight in the  $K_a$  and the  $K_i$  for the  $PGB_x$  fractions separated by dialysis.

TABLE I

The Analysis of  $PGB_x$  Fractions  
Separated by Dialysis at pH 6.5

Fraction	Weight (mg)	In Vitro $PGB_x$ Effect			
		Activation		Inhibition	
		$K_a$	Total $K_a$	$K_i$	Total $K_i$
$PGB_x$	116.4	1.0	116.4	1.0	116.4
Rententate	105.3	0.99	104.2	1.063	111.6
Dialysate	9.5	1.08	10.3	0.25	2.4
% Recovery	98.6		98.4		97.9

The dialysis technique used in this study is a relatively simple and rapid method. However, it does not separate quantitatively the  $PGB_x$  activator from the  $PGB_x$  inhibitor, since only a small portion (5) of the total activator is obtained relatively "free" of the inhibitor factor. Conceivably the activator and inhibitor factors exist as heterogeneous mixtures of different molecular weight substances which make up the  $PGB_x$  complex. The activator factor range of molecular weights falls within the molecular size separable by dialysis. On the other hand the inhibitor factor molecular weight range is above this cut off point and consequently is not dialyzable. Because of the wide range in molecular weights of both factors, the separation is not sharp, and some of the inhibitor factor does pass through the membrane, as evidenced by the drop in the phosphorylation level at the high concentrations of the dialyzed  $PGB_x$  (figure 2).



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